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Carboxylate Anion Binding in the Cyclohexaamylose Cavity: A Steric and Electronic Evaluation

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This investigation focuses on the steric and electronic requirements for the binding of carboxylate anions in the cyclohexaamylose cavity. The geometries and thermodynamic stabilities of the 3,5-dimethyl-4-hydroxybenzoic acid, 3,5-dimethyl-4-hydroxycinnamic acid, and 3,5-dimethyl-4-hydroxyhydrocinnamic acid cyclohexaamylose complexes are evaluated at pH = 7.60 and 12.00. Results indicate that the carboxylate anions of 3,5-dimethyl-4-hydroxycinnamic acid and 3,5-dimethyl-4-hydroxyhydrocinnamic acid bind in the cyclohexaamylose cavity at both pH = 7.60 and 12.00. In addition, the dependence of the stability of the resulting complexes on pH is shown to be marginal. These findings suggest that if a carboxylate anion is to bind in the cyclohexaamylose cavity, it must be able to adopt a position in the cavity which allows for at least partial solvation of charge.

INTRODUCTION

The cycloamyloses are a group of cyclic oligosaccharides containing from 6 to 12 α-1,4-linked glucopyranose units (*1–3*). In aqueous solution, these torus-shaped systems have been shown to complex a variety of guest molecules within their hydrophobic interiors, and in some cases, to catalyze reactions of the complexed substrates. Because of these phenomena, a great deal of effort has been expended in developing the cycloamyloses as enzyme-active site models. The thrust of these studies has been to expand their spectrum as catalysts (*4*, *5*) and to improve their catalytic abilities by chemical modification (*6*, *7*). Endeavors in both these areas have met with great success. However, little use has been made of these compounds as models for the study of the steric and electronic features which regulate enzyme–substrate binding. The cycloamyloses offer a number of advantages for such studies: (1) Cycloamylose substrate binding constants, as well as the thermodynamic parameters for inclusion, can be measured easily and accurately with a variety of different techniques. (*8–11*). (2) The systems can be chemically modified with some facility (*12–14*). (3) Because of the symmetry of the cycloamyloses, their ^{13}C and ^{1}H nmr spectra are very simple (*11*, *13–15*). It is therefore possible, by observing complexation-induced changes in ^{13}C and ^{1}H nmr spectra of both the cycloamylose and substrate, to determine the geometry of the complex, as well as the tightness of complexation (*11*, *13*, *14*, *16*).

In a recent series of studies focusing on the origins of cycloamylose substrate binding energy, we demonstrated that both sodium *p*-nitrophenolate and *p*-nitrophenol

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penetrated the cyclohexaamylose cavity nitro group first at the 2,3-hydroxyl side (*13*). In the course of this investigation, we observed that the charged phenolates always bound in the cavity more tightly than the corresponding neutral phenols (*11*). This observation was in keeping with our ideas about the importance of dipolar interactions in cycloamylose substrate binding. However, because of the strong delocalization of charge in the *p*-nitrophenolates studied, the importance of the position of substrate charge in regulating each complex's geometry and stability remained unclear. We there-

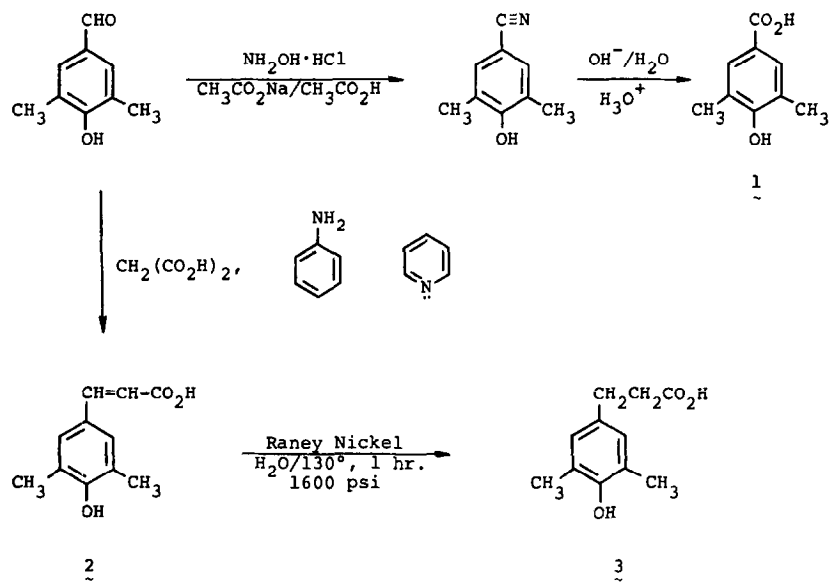


FIG. 1. Synthesis of 3,5-dimethyl-4-hydroxyphenylcarboxylic acids from 3,5-dimethyl-4-hydroxybenzaldehyde.

fore decided to investigate a less delocalized substrate whose cycloamylose binding was much more charge sensitive.

Benzoic acid binds in the cyclohexaamylose cavity 87 times more tightly than the corresponding benzoate anion (*16*), displaying a charge dependence opposite that observed in the *p*-nitrophenol, *p*-nitrophenolate systems. ^{1}H nmr studies demonstrated that benzoic acid bound in the cyclohexaamylose cavity carboxyl group first (*16*). For the sodium benzoate complex, this geometry would be unstable due to the energy required to move the carboxylate anion into the cycloamylose's hydrophobic interior. Although ^{1}H nmr evidence did suggest that the carboxylate anion could be penetrating the cavity, the evidence was not definitive. In this investigation, we examine carboxylate anion binding in the cyclohexaamylose cavity more closely. The electronic and steric features of the substrate necessary for the carboxylate anion to bind in the cyclohexaamylose cavity are evaluated. The system is designed to evaluate 1 : 1 and not 2 : 1 complexes (*17*, *18*).

The substrates investigated: 3,5-dimethyl-4-hydroxybenzoic acid (1), 3,5-dimethyl-4-hydroxycinnamic acid (2), and 3,5-dimethyl-4-hydroxyhydrocinnamic acid (3) were

chosen for four reasons: (1) The substrates bind in a limited number of geometries. (2) The limits of the depth of cavity penetration in each substrate orientation is assessable. (3) The charge density on the substrate molecules is variable; the charge on the carboxylate anion can be intensified. (4) The complexes formed can only be simple AB complexes.

The substrates were all synthesized from 3,5-dimethyl-4-hydroxybenzaldehyde (Fig. 1). Except for the parent acid, the synthesis did not present any problems. The acid had to be generated via the nitrile because of the low yield realized from permanganate or chromic acid oxidations of the aldehyde.

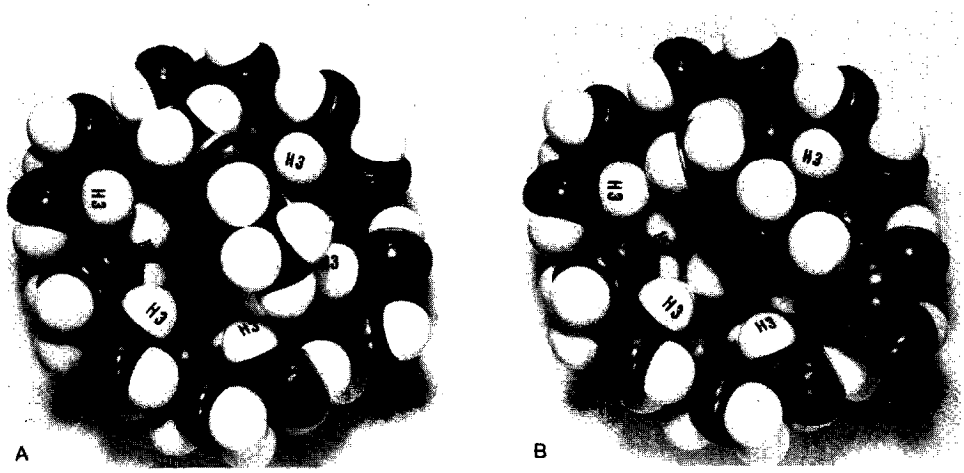


FIG. 2. CPK space-filling models of the two possible orientations of 3,5-dimethyl-4-hydroxycinnamic acid in the cyclohexaamylose cavity at the 2,3-hydroxyl side. (A) The substrate's carboxyl group is penetrating the cavity. (B) The substrate's methyl and hydroxyl groups are penetrating the cavity.

These substrates have two sterically allowed binding geometries at the 2,3-hydroxyl side of the cyclohexaamylose, one with the carboxylate sitting in the cavity (A), and one with the 4-hydroxyl and a methyl group sitting in the cavity (B) (Fig. 2). It is important to recognize that geometry B places the 4-hydroxyl group in intimate contact with the cyclohexaamylose cavity wall. Furthermore, in geometry A, because of the methyl groups in the 3 and 5 positions of the substrates, the depth to which each substrate's carboxylate can penetrate the cavity is limited.

The contribution of each of these geometries to substrate binding should be reflected by the pH dependence of the free energy of formation. At a pH where only the carboxylate anions are present, binding geometries A and B for substrates 2 and 3 are both feasible on the basis of anion solvation. In geometry A, the carboxylate anions of substrates 2 and 3 can reach the back of the cavity where they may be hydrogen bonded to the 6-CH's or partially solvated. However, the carboxylate anion of substrate 1 cannot penetrate as deeply as those of substrates 2 and 3 and thus cannot be solvated as effectively. Therefore, geometry B appears to be more favorable in this case. At a high pH, where the substrates are present as dianions, geometry B should be highly

unfavorable for all three substrates due to the poor solvation of the phenolate anion while inside the cyclohexaamylose cavity.

In this study we demonstrate that of the three cycloamylose substrate complexes investigated, only the stability of the 3,5-dimethyl-4-hydroxybenzoic acid complex is very sensitive to pH. This suggests that carboxylate anions can bind in the cyclohexaamylose cavity when in a position to be at least partially solvated.

EXPERIMENTAL SECTION

Materials. The cyclohexaamylose was obtained from Aldrich Chemical Company. The 3,5-dimethyl-4-hydroxybenzaldehyde was prepared by a literature method (*19*). The 3,5-dimethyl-4-hydroxybenzoic, 3,5-dimethyl-4-hydroxycinnamic, and 3,5-dimethyl-4-hydroxyhydrocinnamic acids were synthesized by the procedures outlined below.

Preparation of 3,5-dimethyl-4-hydroxybenzoic acid. 3,5-Dimethyl-4-hydroxybenzaldehyde (*19*) (6 g, 0.040 mol) was added to a solution of anhydrous sodium acetate (4.0 g, 0.049 mol) and hydroxylamine · HCl (3.4 g, 0.049 mol) in glacial acetic acid (25 ml) and refluxed for 14 hr. The reaction mixture was reduced *in vacuo*, added to 250 ml of H_2O, and neutralized with $NaHCO_3$. This mixture was extracted 3 × 100 ml ether and the ether extract washed with 2 × 100 ml 1% aqueous NaOH. The NaOH wash was refluxed for 12 hr after the addition of 30 g of NaOH. The solution was acidified with 50% HCl and extracted 5 × 100 ml ether. The aqueous ether extract was washed 5 × 25 ml 1% NaOH solution. Acidification of the basic wash with 50% HCl followed by filtration and recrystallization from water gave 4.0 g (61%) of the desired product: mp 124°C (lit. 123–124°C) (*19*); nmr (d_6-DMSO) δ 2.20 (s, 6H), 5.75 (s, 1H), and 7.55 (s, 2H); ir (KBr) 3430, 1650, 1115, and 1020 cm^{-1}.

Anal. Calcd for $C_9H_{10}O_3$: C, 65.05; H, 6.07. Found: C, 65.05; H, 6.05.

Preparation of 3,5-dimethyl-4-hydroxycinnamic acid. 3,5-Dimethyl-4-hydroxybenzaldehyde (10 g, 0.073 mol) was added to a stirred solution of malonic acid (7.5 g, 0.072 mol) and aniline (0.420 ml, 0.004 mol) in pyridine (20 ml). The reactants were stirred at 65°C for 7.5 hr. After cooling, 250 ml of a 20% HCl solution was added. The solid was filtered and recrystallized from MeOH/H_2O, affording 11.3 g (88.3%) of *trans*-3,5-dimethyl-4-hydroxycinnamic acid: mp 205–206°C; nmr (d_6-DMSO) δ 2.20 (s, 6H), 5.20 (d, 1H, J = 16.0 Hz), 7.21 (s, 2H) and 7.40 (d, 2H, J = 16.0 Hz); ir (KBr) 3400, 1650, 1142, and 1010 cm^{-1}.

Anal. Calcd for $C_{11}H_{12}O_3$: C, 68.74; H, 6.29. Found: C, 68.70; H, 6.37.

Preparation of 3,5-dimethyl-4-hydroxyhydrocinnamic acid. 3,5-Dimethyl-4-hydroxycinnamic acid (2.4 g, 0.012 mol) was added to 250 ml H_2O and the pH adjusted to 7.0. To this solution was added 500 mg of Raney nickel. The reduction was run at 130°C under 1600 psi of H_2 pressure for 1.5 hr. The solution was acidified and the resulting white crystalline solid was filtered. Recrystallization from $CHCl_3$ afforded an almost quantitative yield of the 3,5-dimethyl-4-hydroxyhydrocinnamic acid: mp 109–110°C; nmr ($CDCl_3$) δ 2.20 (s, 6H), 2.65 (m, 4H), and 6.75 (s, 2H); ir (KBr) 3425, 1690, 1280, and 1145 cm^{-1}.

Anal. Calcd for $C_{11}H_{14}O_3$: C, 68.02; H, 7.27. Found: C, 68.07 and H, 7.36.

Determination of 1st and 2nd pK_a constants for 3,5-dimethyl-4-hydroxyphenylcarboxylic acids. The first (carboxylate) pK_a for each compound was determined by titration of a 1.0×10^{-3} *M* solution of the acid with a 1.0×10^{-2} *M* solution of sodium hydroxide. The pK_a was taken as the pH at the volume of titrant equal to half the volume required to reach the first endpoint. The second (phenolate) pK_a for each compound was determined by titration of a 0.050 *M* solution of the carboxylate with a 0.250 *M* solution of sodium hydroxide. The pK_a was taken as the pH at the volume of titrant equal to three-fourths the total volume required to reach the second endpoint. All solutions were prepared in distilled water which had been boiled to remove CO_2. All titrations were performed under nitrogen at a temperature of 25.0 ± 0.2°C. The pH was followed using a Radiometer PHM 64 pH meter.

Sample preparation for determination of dissociation constants by ultraviolet method. Solutions of cyclohexaamylose were prepared in disodium phosphate buffer (pH = 7.60 ± 0.02, I = 0.5) and in trisodium phosphate buffer (pH = 12.00 ± 0.02, I = 0.5). In each case, the pH was adjusted with phosphoric acid.

The samples of 3,5-dimethyl-4-hydroxybenzoic, 3,5-dimethyl-4-hydroxycinnamic, and 3,5-dimethyl-4-hydroxyhydrocinnamic acids were made up in the same pH = 7.60 and 12.00 phosphate buffers. In order to prevent decomposition, all substrate solutions were kept out of light throughout preparation and use.

Sample preparation for nuclear magnetic resonance. The cyclohexaamylose hydroxyl protons were exchanged for deuterium by lyophilizing 600 mg of the carbohydrate from 40 ml of D_2O three times. This helped to minimize the HOD in the final sample. The buffer solutions were made up with anhydrous Na_3PO_4 in D_2O and the pD adjusted with deuterophosphoric acid. The final pD values were 7.60 ± 0.02, and 12.00 ± 0.02, I = 0.5 for each. These pD values were obtained by adding 0.4 to the pH meter reading, using an electrode which had been standardized with both pH = 10.00 ± 0.01 and pH = 4.01 ± 0.01 buffer in H_2O and then rinsed with D_2O (*18*). All substrate solutions were kept from light to avoid decomposition.

Determination of binding constants by the second-order ultraviolet spectra method. Complexation-induced changes in each substrate's electronic spectrum were measured as a function of increasing cyclohexaamylose substrate ratios using a Cary Model 14 recording spectrophotometer with the cell compartment thermostated at 25 ± 1°C.

In all cases, dual compartment cells were used, equal amounts of substrate and cycloamylose solutions being placed in either side of each cell. Spectra were taken both before and after inverting and shaking one of the cells. In this manner, the changes in substrate absorbance caused by complexation were measured directly. The substrate concentrations were held constant at 8.00×10^{-5}, 4.00×10^{-5}, and 6.00×10^{-4} *M* at pH = 7.60, and 6.00×10^{-5}, 4.00×10^{-5}, and 3.00×10^{-4} *M* at pH = 12.00 for the 3,5-dimethyl-4-hydroxybenzoic, 3,5-dimethyl-4-hydroxycinnamic, and 3,5-dimethyl-4-hydroxyhydrocinnamic acids, respectively. At both pH = 7.60 and pH = 12.00, the cycloamylose concentration was varied from 1.56×10^{-3} to 5.00×10^{-2} *M*.

The data were treated according to a modified Hildebrand–Benesi equation by plotting $C_0/\Delta\text{Abs} + S_0\Delta\text{Abs}/\Delta\varepsilon^2$ vs $C_0 + S_0$ to obtain a straight line with slope equal to $1/S_0\Delta\varepsilon$ and intercept equal to $K_D/S_0\Delta\varepsilon$ (see Appendix A). Linear least-squares analyses were performed on all data, and only K_D values with high correlation coefficients (>0.98) were accepted.

Determination of cycloamylose substrate dissociation constants by ^1H nmr. ^{1}H-Pulsed Fourier transform nmr spectra (220.02 MHz) were obtained on a Varian Supercon spectrometer at 25 ± 1°C. The changes in chemical shifts of the substrate protons, as well as the changes in chemical shifts of the cyclohexaamylose protons, were measured as a function of changing cyclohexaamylose substrate ratios. The 3,5-dimethyl-4-hydroxybenzoic acid, cinnamic acid, and hydrocinnamic acid substrates were made up in phosphate buffer at pD = 7.60 ± 0.02 and pD = 12.00 ± 0.02; I = 0.5. For the titrations of the substrates with cyclohexaamylose at pD = 7.60 and 12.00, the concentrations of the substrates were held constant at 0.006 *M* in every case. The cyclohexaamylose concentrations varied between 1.0×10^{-2} and 7.0×10^{-2} *M* in the cases of the 3,5-dimethyl-4-hydroxybenzoic and 3,5-dimethyl-4-hydroxyhydrocinnamic acids at pD = 7.60 and pD = 12.00 and the 3,5-dimethyl-4-hydroxycinnamic acid at pD = 12.00, while in the case of the 3,5-dimethyl-4-hydroxycinnamic acid at pD = 7.60, the concentration varied between 1.0×10^{-3} and 3.5×10^{-2} *M*.

For the titrations of cyclohexaamylose with the 3,5-dimethyl-4-hydroxyphenylcarboxylic acids at pD = 7.60 and 12.00, the concentration of the cyclohexaamylose was held constant at 0.005 *M* and the concentrations of the substrates varied between 0.001 and 0.100 *M*.

The data were treated according to a modified Hildebrand–Benesi equation by plotting $C_0/\Delta\delta + S_0\Delta\delta/Q^2$ vs $C_0 + S_0$ to obtain a straight line with slope equal to $1/Q$ and an intercept equal to K_D/Q (see Appendix A). Linear least-squares analyses were performed on all data, and only K_D values with high correlation coefficients (>0.98) were accepted.

^1H homonuclear Overhauser enhancements (NOE's) for the monosodium 3,5-dimethyl-4-hydroxycinnamate cyclohexaamylose complex. A solution of sodium 3,5-dimethyl-4-hydroxycinnamate (0.05 *M*) and cyclohexaamylose (0.10 *M*) in Na_3PO_4 buffer (pD = 7.60; I = 0.5) was prepared. ^{1}H homonuclear Overhauser enhancements (NOE's) were determined from ^{1}H-pulsed Fourier transform nmr spectra (100.1 MHz) obtained on a Varian FT XL-100 spectrometer at 25 ± 1°C. Enhancements are reported as the percentage difference in integrated intensity of the resonance being observed when the second radiofrequency (rf) was applied first at the resonance frequency to be irradiated, and then set to a vacant region of the spectrum. In the 100.1 MHz NOE experiments, the data for both spectra (on resonance and off resonance) were obtained concurrently by alternating data acquisition after each pulse sequence. The irradiating rf was allowed to maintain the first frequency for the entire pulse sequence before switching to the second frequency.

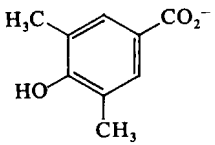
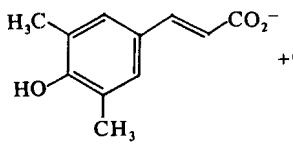
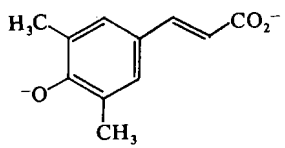
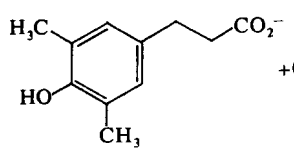
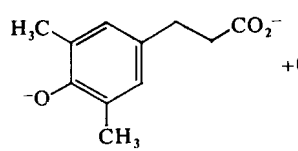
RESULTS

Determination of Free Energies of Formation for Cycloamylose Substrate Complexes by ^1H nmr

The changes in the ^{1}H nmr spectra experienced by both the host and guest molecules on complexation have made it possible to verify that the free energies of formation determined in these studies represent binding of the substrate inside the cyclohexaamylose cavity.

TABLE 1

FREE ENERGIES OF FORMATION FOR THE VARIOUS CYCLOAMYLOSE SUBSTRATE COMPLEXES AND Q VALUES (ppm) FOR THE SUBSTRATE'S PROTONS[a]

Substrate	Aromatic Q	Methyl Q	α Vinyl Q	β Vinyl Q	α Methylene Q	β Methylene Q	ΔG_f (kcal mol^{-1})
	+0.127 ± 0.002	+0.014 ± 0.005	—	—	—	—	$\Delta G_f^{nmr} = -1.71 \pm 0.25$
	+0.130 ± 0.005	+0.127 ± 0.010	—	+0.096 ± 0.002	—	—	$\Delta G_f^{nmr} = -3.43 \pm 0.15$ $\Delta G_f^{uv} = -2.96 \pm 0.12$
	Too broad	Too broad	−0.057 ± 0.002	Too broad	—	—	$\Delta G_f^{nmr} = -1.85 \pm 0.47$ $\Delta G_f^{uv} = -1.94 \pm 0.28$
	+0.030 ± 0.002	+0.038 ± 0.001	—	—	—	—	$\Delta G_f^{nmr} = -2.49 \pm 0.20$ $\Delta G_f^{uv} = -2.10 \pm 0.30$
	+0.098 ± 0.002	+0.045 ± 0.001	—	—	−0.011 ± 0.001	−0.057 ± 0.002	$\Delta G_f^{nmr} = -1.56 \pm 0.40$ $\Delta G_f^{uv} = -1.57 \pm 0.94$

[a]As determined from the data analysis described in Appendix A. Downfield shifts are indicated by positive Q values.

From the observed changes in the chemical shifts for both the cyclohexaamylose host and the respective guest molecules at pD = 7.60 and 12.00, as well as from the known forward and reverse rate constants for similar cyclohexaamylose substrate association equilibrium (e.g., $5.2 \times 10^8\ M^{-1}\ sec^{-1}$ and $1.3 \times 10^5\ sec^{-1}$, respectively, for the cyclohexaamylose sodium *p*-nitrophenolate complex) (*21*), it is clear that the system is in the nmr chemical-shift fast-exchange limit (*22*). This means that the various substrate proton resonances appear at the average of the chemical shifts of substrate free and bound in each possible orientation to cyclohexaamylose, weighted by the fractional population of the substrate molecule in each environment. Of course, the same is true for the cyclohexaamylose molecule with each of its resonances occurring at its fast exchange position, weighted by the fraction of empty cyclohexaamylose molecules and the fraction of cyclohexaamylose molecules having guests.

The free energies of formation, ΔG_f's, as well as the maximum chemical shift changes, Q values (see Appendix A), of 3,5-dimethyl-4-hydroxybenzoic acid, 3,5-dimethyl-4-hydroxycinnamic acid, and 3,5-dimethyl-4-hydroxyhydrocinnamic acid cyclohexaamylose complexes at both pD = 7.60 and pD = 12.00 are given in Table 1. The induced chemical shift changes in the ^1H nuclear magnetic resonance spectra of the substrate were measured as a function of an increasing cycloamylose substrate ratio at both pD values. The data gave excellent correlation coefficients to straight-line plots of $\Delta\delta$ vs mole fraction bound, indicating that an A + B $\rightleftharpoons$ AB equilibrium model was likely (*11*).

Cyclohexaamylose Binding of 3,5-Dimethyl-4-hydroxybenzoic Acid at pD (pH) = 7.60 and pD (pH) = 12.00

At pD = 7.60, the ΔG_f for the 3,5-dimethyl-4-hydroxybenzoic acid cyclohexaamylose complex determined by ^1H nmr was -1.71 ± 0.25 kcal mol^{-1}. The ΔG_f was determined from the chemical shift changes in the aromatic protons ($Q = +0.127 \pm 0.002\ \delta$) because the changes observed for the methyl protons ($Q \approx +0.014 \pm 0.005$) were very small. There was no evidence for the binding of 3,5-dimethyl-4-hydroxybenzoic acid at pD = 12.00. The maximum chemical shift change induced in the substrate at pD = 12.00 was no greater than $\pm 0.004\ \delta$ at a cycloamylose substrate molar ratio of 12.

At pH = 7.60, complexation-induced changes in the electronic spectra of the 3,5-dimethyl-4-hydroxybenzoic acid were too small to accurately determine a free energy of formation using the second-order ultraviolet spectra method. At pH = 12.00, there was no evidence for binding at all.

Cyclohexaamylose Binding of 3,5-Dimethyl-4-hydroxycinnamic Acid at pD (pH) = 7.60 and pD (pH) = 12.00

The free energies of formation for the 3,5-dimethyl-4-hydroxycinnamic acid at pD = 7.60 and 12.00 as determined by ^1H nmr changes induced in the substrate by cyclohexaamylose are -3.43 ± 0.15 kcal mol^{-1} and -1.85 ± 0.47 kcal mol^{-1}, respectively. Except for the protons α to the carboxylate anion, the chemical shift changes observed in the spectra of 3,5-dimethyl-4-hydroxycinnamic acid when complexed at pD = 7.60 are all of the same order of magnitude ($Q \approx +0.130\ \delta$). Although the α protons are shielded at both pD = 7.60 and pD = 12.00, the Q value at pD = 7.60 could not be

accurately determined because the induced changes were very small. The α carbon is apparently very sensitive to the total charge on the substrate. In going from pD = 3.00 to pD = 7.60 the α proton of the substrate alone shifts only −0.010 δ while on going from pD = 7.60 to pD = 12.00 the α proton shifts −0.190 δ.

The 3,5-dimethyl-4-hydroxycinnamic acid resonances at pD = 12.00 suffer considerable line broadening as the substrate becomes bound. Broadening was much more substantial for the aromatic and methyl protons than for the protons α to the carboxylate anion. An accurate assessment of broadening in the β proton signal could not be made since the aromatic and β proton resonances overlap.

Free energies of formation for the 3,5-dimethyl-4-hydroxycinnamic acid cycloamylose complex at pH = 7.60 and pH = 12.00 were also determined using the second-order ultraviolet spectra method ($\Delta G_f = -2.96 \pm 0.12$ kcal mol^{-1}, pH = 7.60; $\Delta G_f = -1.94 \pm 0.28$ kcal mol^{-1}, pH = 12.00). Treatment of the data according to the modified Hildebrand–Benesi equation (Appendix A) gave excellent straight-line fits, again indicating an A + B $\rightleftharpoons$ AB equilibrium.

Cyclohexaamylose Binding of 3,5-Dimethyl-4-hydroxyhydrocinnamic Acid at pD (pH) = 7.60 and pD (pH) = 12.00

The complexation-induced changes in the ^{1}H nmr spectra of this complex were again measured as a function of an increasing cycloamylose substrate ratio. The chemical shift changes of both the methyl and aromatic protons of 3,5-dimethyl-4-hydroxyhydrocinnamic acid were easily followed. Although the methylene protons α and β to the carboxylate anion at both pD = 7.60 and pD = 12.00 are deshielded upon cyclohexaamylose complexation, they undergo substantial line broadening making accurate chemical shift measurement difficult. The ΔG_f's for 3,5-dimethyl-4-hydroxyhydrocinnamic acid as determined from chemical shift changes induced in the aromatic and methyl protons were −2.49 ± 0.20 kcal mol^{-1} at pD = 7.60 and −1.56 ± 0.40 kcal mol^{-1} at pD = 12.00. At pD = 7.60, the Q values for the aromatic and methyl groups are +0.030 ± 0.002 δ and +0.038 ± 0.001 δ, respectively. However, at pD = 12.00, the Q value obtained for the aromatic protons, +0.098 ± 0.002 δ, is 2.2 times that of the methyl protons, +0.045 ± 0.001 δ.

Free energies of formation for the 3,5-dimethyl-4-hydroxyhydrocinnamic acid cyclohexaamylose complex at pH = 7.60 and pH = 12.00 as determined by the second-order ultraviolet method were −2.10 ± 0.30 kcal mol^{-1} and −1.57 ± 0.94 kcal mol^{-1}, respectively. Again when data were treated according to the modified Hildebrand–Benesi equation, excellent straight line fits resulted, indicating A + B $\rightleftharpoons$ AB equilibrium at both pH = 7.60 and 12.00.

Effects of Substrate Binding on the Cyclohexaamylose ^{1}H NMR Spectra

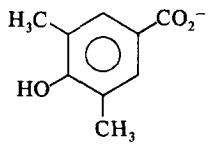
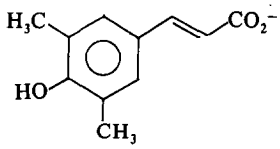
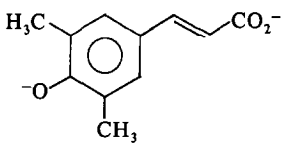
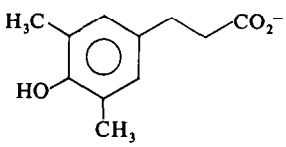
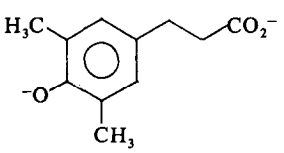
The spectrum of free cycloheptaamylose as well as the spectra for a variety of cycloheptaamylose aromatic complexes were assigned by Demarco and Thakker (*23*). They demonstrated that upon complexation of any of several aromatic guests, the cycloheptaamylose H-5, and to a lesser degree H-3, resonances are shifted upfield because of the diamagnetic anisotropy of the included aromatic guests. We observed similar shifts of the cycloheptaamylose H-5 and H-3 protons on complexation of both *p*-nitrophenol and sodium *p*-nitrophenolate (*11*, *13*). However, we demonstrated that only the H-3

protons were shielded in the cyclohexaamylose complex, i.e., the substrate only partially penetrated the cavity.

^{1}H nmr spectra (220.02 MHz) of cyclohexaamylose were obtained for 3,5-dimethyl-4-hydroxybenzoic acid, 3,5-dimethyl-4-hydroxycinnamic acid, and 3,5-dimethyl-4-

TABLE 2

Q VALUES (ppm) FOR THE CYCLOAMYLOSE H-3 AND H-5 PROTONS AS DETERMINED FROM THE RELATIONSHIP $Q = \Delta\delta_{obs}$/MOLE FRACTION BOUND

Substrate	$Q_{H\text{-}3}$	$Q_{H\text{-}5}$
	−0.060 ± 0.008	−0.029 ± 0.004
	−0.134 ± 0.033	+0.135 ± 0.021
	−0.143 ± 0.046	+0.188 ± 0.029
	−0.058 ± 0.012	+0.112 ± 0.018
	−0.040 ± 0.010	+0.165 ± 0.026

hydroxyhydrocinnamic acid–cyclohexaamylose complexes at both pD = 7.60 and 12.00 as a function of increasing substrate cyclohexaamylose ratios. Because of the difficulty in following specific cyclohexaamylose resonances as the host molecules become more bound, Q values were determined from the relationship $Q = \Delta\delta_{obs}$/mole fraction bound. The values are listed in Table 2. Assuming a ΔG_f of -1.71 ± 0.25 kcal mol^{-1}, the downfield shifts (Q values) determined for the cyclohexaamylose's H-3

and H-5 protons on complexation with 3,5-dimethyl-4-hydroxybenzoic acid at pD = 7.60 are -0.060 ± 0.008 δ and -0.029 ± 0.004 δ, respectively.

The titration of cycloamylose with 3,5-dimethyl-4-hydroxycinnamic acid at pD = 7.60 yielded Q values of -0.134 ± 0.033 δ and $+0.135 \pm 0.021$ δ for the H-3 and H-5 protons, respectively. At pD = 12.00 the 3,5-dimethyl-4-hydroxycinnamic acid titration of cycloamylose generated Q values of -0.143 ± 0.046 δ and $+0.188 \pm 0.029$ δ, respectively. It is clear that the Q values obtained for the H-3 and H-5 protons in the 3,5-dimethyl-4-hydroxycinnamic acid–cycloamylose complexes at both pD = 7.60 and 12.00 are either very close to or within experimental error of one another. Similar trends were observed for shift changes induced in the cycloamylose's H-3 and H-5 protons by 3,5-dimethyl-4-hydroxyhydrocinnamic acid at pD = 7.60 and 12.00. These are -0.058 ± 0.012 δ and $+0.112 \pm 0.018$ δ at pD = 7.60 and -0.040 ± 0.010 δ and $+0.165 \pm 0.026$ δ at pD = 12.00, respectively. Again the values were very close or within experimental error of each other. Finally, at pD = 12.00 the cyclohexaamylose resonances are considerably broadened as the cyclohexaamylose becomes bound with 3,5-dimethyl-4-hydroxycinnamic acid.

Intermolecular Nuclear Overhauser Enhancement

A ^{1}H homonuclear Overhauser experiment was done on the 3,5-dimethyl-4-hydroxycinnamic acid–cyclohexaamylose complex (pD = 7.60) at 100.1 MHz. Substantial enhancements in the integrated intensity of the protons β to the carboxylate anion and the aromatic protons, as well as the protons α to the carboxylate anion, were observed upon saturation of the cyclohexaamylose resonances upfield of the HOD resonance. For a sample in which 3,5-dimethyl-4-hydroxycinnamic acid (pD = 7.60) was 94% bound by cyclohexaamylose, the measured enhancement was $13.3 \pm 1.0\%$ for the aromatic and β vinyl protons together and $9.8 \pm 2.8\%$ for the α vinyl protons.

DISCUSSION

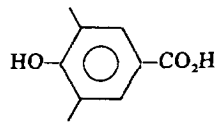
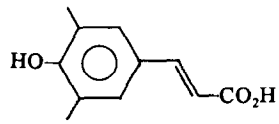
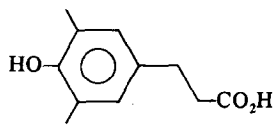
Charge Delocalization in the 3,5-Dimethyl-4-hydroxyphenylcarboxylates

The pK_a values (Table 3) for the substrate acids confirm the importance of charge delocalization in anion stabilization. The second pK_a of 3,5-dimethyl-4-hydroxybenzoic acid, 9.61, is 0.28 pK_a units below that of phenol, suggesting some delocalization of charge. These data imply that for 3,5-dimethyl-4-hydroxybenzoic acid at pH = 12.00, there is less charge on the hydroxyl oxanion than in simple sodium phenolate, but more charge on the carboxylate than in sodium benzoate. Due to the increased charge build-up on this carboxylate anion, desolvation of this anion and insertion into the cycloamylose cavity should require more energy than in the case of the parent sodium benzoate.

This same kind of delocalization exists in 3,5-dimethyl-4-hydroxycinnamic acid, but is absent in 3,5-dimethyl-4-hydroxyhydrocinnamic acid. The second pK_a of the hydroxycinnamic acid, 9.65, is 0.24 pK_a units lower than the pK_a of phenol. However, the second pK_a of the hydroxyhydrocinnamic acid, 10.77, is 0.88 pK_a units greater than the pK_a of phenol. These data suggest that for 3,5-dimethyl-4-hydroxycinnamic acid at

TABLE 3

FIRST AND SECOND pK_a VALUES FOR THE SUBSTRATE ACIDS

Compound	1st pK_a	2nd pK_a
	4.63	9.61
	4.70	9.65
	4.87	10.77

pH = 12.00, there is less charge on the phenolate oxanion than on the oxanion of sodium phenolate, but more charge on the carboxylate than on the carboxylate of sodium cinnamate.

Substrate-Induced Changes in Cyclohexaamylose ^1H nmr Spectra

This segment of the discussion is separated into four sections. The first section is an overview of possible cycloamylose substrate geometries, their interconversions, and theoretical effects on the resonances of the cycloamylose's H-3 and H-5 protons. Each of the remaining three sections is devoted to the specific effects of one of the three substrates on the cyclohexaamylose's ^1H nmr spectrum.

The substrates can bind in the cyclohexaamylose cavity in either of two different orientations: carboxylate anion first (geometry A) or methyl and hydroxyl first (geometry B) (Fig. 2). Binding may occur exclusively in one of these two orientations or, alternatively, it can be bimodal with a certain mole fraction of the substrate bound in each orientation. In geometry A, the H-5 protons are in intimate contact with the substrate's carboxylate or carboxylate side chain. In geometry B, the H-5 protons do not make contact with the substrate. A consequence of this is that for the same mole fraction of cycloamylose bound, the H-5 protons should be shifted more, i.e., the Q values should be more positive in geometry A than in geometry B.

The stability of a particular cycloamylose substrate geometry at a given pH is determined by the stability of the substrate anion in that geometry. When the substrates bind in the cavity at pH = 7.60, the carboxylate anions are more completely solvated in geometry B than in geometry A. However, the carboxylate anion can be at least partially solvated in geometry A by solvent water in the cavity. Furthermore, as the

carboxylate side chain increases in length, its interaction with the cavity improves binding, and brings the carboxylate anion closer to the back of the cavity where it can be more effectively solvated. At pH = 12.00, geometry B is likely to be of high energy since in this orientation the phenolate oxanion cannot be solvated. This means that in going from mono- to dianions, if the substrate is binding in geometry A, neither the free energy of formation nor the Q values for the cycloamylose's H-3 and H-5 protons should change significantly. However, if the substrate is bound in geometry B, the free energy of formation should increase drastically while the Q values should still remain the same. If a certain mole fraction is bound in each geometry, i.e., binding is bimodal, the apparent free energy of formation should increase along with the Q values for the cycloamylose's H-5 protons.

The expected magnitude of such an increase in the apparent free energy of formation can be predicted from our previous studies of the benzoic acid and sodium benzoate cyclohexaamylose complexes (*16*). The free energies of formation for these complexes are -3.96 ± 0.07 kcal mol^{-1} and -1.35 ± 0.21 kcal mol^{-1}, respectively (*16*). It is clear that when benzoic acid's neutral carboxyl group, which has been shown definitively to lie within the cyclohexaamylose cavity, becomes charged, the dissociation constant of the resulting complex increases almost a 100-fold. Furthermore, it is important to recognize that even if the sodium benzoate is also binding in the cyclohexaamylose cavity carboxylate first, the anion can be at least partially solvated by water molecules at the back of the cavity. Our free energy of solution studies clearly indicated that if the carboxylate anion were not solvated at all, the free energy of formation of the sodium benzoate complex would be some 8.73 kcal mol^{-1} less favorable than that for the benzoic acid complex (*16*). In geometry B at pH = 12.00, the phenolate oxanion binding in the cyclohexaamylose cavity cannot be solvated at all. Therefore, at pH = 12.00, the difference in ΔG_f's between geometries A and B should be well in excess of 6 kcal mol^{-1}. The below data is in accord with these predictions.

3,5-Dimethyl-4-hydroxybenzoic Acid

At pH (pD) = 7.60, I = 0.5, 3,5-dimethyl-4-hydroxybenzoic acid binds in the cyclohexaamylose cavity shielding both the H-3 and H-5 methine protons ($\Delta G_f^{nmr} = -1.71 \pm 0.25$ kcal mol^{-1}). The Q values for the H-3 and H-5 protons are -0.060 ± 0.008 δ and -0.029 ± 0.004 δ, respectively. The shielding of the H-3 proton can be attributed to its sitting in the magnetic field of the aromatic ring's pi cloud (*24*). Although shielding of the H-5 protons has been observed and discussed for other cycloamylose substrate systems, e.g., sodium 3,5-dimethyl-4-nitrophenolate, the mechanism for this is not yet clear (*16*). Finally, based on the nmr data at pH (pD) = 12.00, I = 0.5, the dianion of 3,5-dimethyl-4-hydroxybenzoic acid does not seem to bind in the cyclohexaamylose at all. This strongly suggests that geometry B is the most favorable substrate orientation at pH (pD) = 7.60 but does not preclude some contribution from geometry A at this pH. At pH (pD) = 12.00, because of the charge delocalization, the carboxylate would be expected to be more unstable in geometry A than simple sodium benzoate. However, due to the small chemical shifts induced in the cyclohexaamylose ^{1}H nmr spectrum by the substrate at pH (pD) = 7.60 and the apparent absence of binding at pH (pD) = 12.00, it is difficult to definitively show anything beyond the fact that this substrate is binding at the 2,3-hydroxyl side of the cavity.

3,5-Dimethyl-4-hydroxycinnamic Acid

At pH (pD) = 7.60, I = 0.5, 3,5-dimethyl-4-hydroxycinnamic acid binds in the cyclohexaamylose cavity fairly tightly ($\Delta G_f^{nmr} = -3.43 \pm 0.15$ kcal mol^{-1}; $\Delta G_f^{uv} = -2.96 \pm 0.12$ kcal mol^{-1}). The Q values for the H-3 and H-5 protons at this pH are -0.134 ± 0.033 δ and $+0.135 \pm 0.021$ δ, respectively. At pH (pD) = 12.00, the substrate binds slightly weaker than at pH (pD) = 7.60 ($\Delta G_f^{nmr} = -1.85 \pm 0.47$ kcal mol^{-1}; $\Delta G_f^{uv} = -1.94 \pm 0.28$ kcal mol^{-1}). At pH (pD) = 12.00, the Q values for the cycloamylose's H-3 and H-5 protons are -0.143 ± 0.046 δ and $+0.188 \pm 0.029$ δ, respectively. At both pH's, the shielding of the H-3 protons can again be attributed to their being in the magnetic field of the aromatic pi cloud. The deshielding of the H-5 methine protons can be attributed to van der Waals's contact shifts between these protons and the substrate's vinyl protons (*16*, *25*).

We recognize the errors are large. However, the differences in free energies of formation and Q values are very small compared to those which would be expected if geometry B were an important component in the overall binding scheme. The small changes observed in the free energies of formation and Q values with pH suggest that significant binding transitions are not occurring and binding of the carboxylate anion in the cavity (geometry A) is the most favorable orientation. In geometry A when the substrate is in the form of the dianion, the phenolate anion is well solvated and the carboxylate is at the back of the cavity in a position to be at least partially solvated. The fact that the substrate was binding in the cycloamylose cavity at its 2,3-hydroxyl side was evidenced by an intermolecular nuclear Overhauser effect.

At pH (pD) = 7.60, when the substrate is 94% bound, irradiation of the cycloamylose H-3 and H-5 methines produces a rather substantial enhancement in the integrated intensities of the aromatic and vinyl protons. The aromatic and β methine protons' multiplet area is increased by $13.3 \pm 1.0\%$; the α methine proton's area by $9.8 \pm 2.8\%$. Although quantitative interpretation of intermolecular NOE's can be complicated, it is simplified since the chemical-shift fast-exchange approximation holds for the present system (*26*). The magnitude of the observed NOE is dependent on the extent to which the nucleus whose resonance is being observed is relaxed by the nucleus being saturated. This relaxation is dependent on the mole fraction of substrate bound, the distance between the interacting protons (r^{-6} dependence), and the substrate residence time (*27*). The lifetime of the species being observed, i.e., the substrate in its bound environment, must be long enough for significant intermolecular relaxation to occur. Considering these dependencies, the magnitude of the intermolecular nuclear Overhauser effect observed suggests rather intimate contact between the cavity's protons and the substrate's vinyl protons.

3,5-Dimethyl-4-hydroxyhydrocinnamic Acid

At pH (pD) = 7.60, I = 0.5, this substrate binds in the cavity slightly weaker than the cinnamic acid under the same conditions ($\Delta G_f^{nmr} = -2.49 \pm 0.20$ kcal mol^{-1}; $\Delta G_f^{uv} = -2.10 \pm 0.30$ kcal mol^{-1}). The Q values for the H-3 and H-5 protons are -0.058 ± 0.012 δ and $+0.112 \pm 0.018$ δ, respectively. The fact that the H-3 protons are less shielded by 3,5-dimethyl-4-hydroxyhydrocinnamic acid than by its cinnamic acid analog at the same pH could be related to the differences in the relative depths of

substrate penetration. Of course, deshielding of the H-5 protons can be explained by a van der Waals contact shift.

The ΔG_f's ($\Delta G_f^{nmr} = -1.56 \pm 0.40$ kcal mol^{-1}; $\Delta G_f^{uv} = -1.57 \pm 0.94$ kcal mol^{-1}) at pH (pD) = 12.00, again suggest the difference in binding between the mono- and dianion is small. The Q values for the cycloamylose H-3 and H-5 protons are -0.040 ± 0.010 δ and $+0.165 \pm 0.026$ δ, respectively. The mechanisms responsible for these shifts are likely to be the same as those described above. Again, as with the 3,5-dimethyl-4-hydroxycinnamic acid, the small changes in Q values and free energies of formation with pH support the importance of geometry A for both the mono- and dianions.

Because the methylene protons of the substrates relax each other, nuclear Overhauser experiments were not attempted with this system. However, the fact that the substrate was binding inside the cycloamylose cavity at the 2,3-hydroxyl side is evidenced by the shielding of the host's H-3 protons and the deshielding of the H-5 protons.

Cyclohexaamylose-Induced Changes in the ^1H nmr Spectra of Substrate Acids

The 3,5-dimethyl-4-hydroxybenzoic acid and 3,5-dimethyl-4-hydroxyhydrocinnamic acid protons were all deshielded on complexation by cyclohexaamylose. These downfield ^1H nmr shifts can be induced in the spectrum of one molecule when binding to another by several physical mechanisms: diamagnetic anisotropy of particular bonds or regions of the host (*28*), van der Waals shifts (*25*), or steric perturbation (*29*). From the limited amount of experimental data available, it is not possible to definitively assign the observed downfield shifts to a particular mechanism.

The changes induced in the ^1H nmr spectrum of 3,5-dimethyl-4-hydroxycinnamic acid on cycloamylose complexation are very different from those observed for the other substrates. The cinnamic acid's α protons are shielded at both pH's. In addition, at pH (pD) = 12.00 they experience substantial line broadening. Prior to this study, we and others have always observed that substrate protons were deshielded on cycloamylose complexation and line broadening, if it occurred, was very slight.

Studies of chemical shift changes of the free substrate protons as a function of pH reveal that the α protons are very sensitive to charge variation in the molecule. In going from pH (pD) = 3.00 to 7.60, the α proton shifts only -0.010 δ. However, on going from pH (pD) = 7.60 to 12.00, the α proton shifts -0.190 δ.

The cycloamlyose-induced shifts in the substrate's α proton at pH (pD) = 7.60 were too small to measure. However, at pH (pD) = 12.00, a Q value of -0.057 ± 0.002 δ was obtained. Again, as with the unbound substrate, the largest changes are occurring at pH (pD) = 12.00. The shielding of the substrate's α protons on cycloamylose complexation suggests that the charge on the α carbon, and therefore on the α proton, is somehow enhanced relative to that on the uncomplexed substrate. This can be related to the poor solvation of charge on the α carbon when inside the host's cavity where no solvent molecules are available.

The linewidths of the 3,5-dimethyl-4-hydroxycinnamic acid at pH (pD) = 12.00 increased significantly with the fraction of substrate bound to cycloamylose. No internal linewidth standard was available since the possibility existed that any small molecule added to the solutions to provide such a standard would compete with the

substrate in binding to the cycloamylose. Thus no perfectly quantitative linewidth comparisons are possible between one sample and the next. However, for the binding of 3,5-dimethyl-4-hydroxycinnamic acid to cyclohexaamylose at pH (pD) = 12.00, it was clear that in each sample the signal area associated with the aromatic and β protons broadened significantly. For example, under the spectrometer conditions used, including an exponential weighting factor for free induction decay of −2.0 sec, the total linewidths of the aromatic, β and α protons are approximately 2 Hz each for a sample of 0.006 *M* 3,5-dimethyl-4-hydroxycinnamic acid (pD = 12.00). Under the same spectrometer conditions, the spectrum of a sample 0.006 *M* in 3,5-dimethyl-4-hydroxycinnamic acid and 0.040 *M* in cyclohexaamylose, i.e., 49% substrate bound to cyclohexaamylose, shows that the aromatic and β proton signals merge providing a single signal 15 Hz in width while the α proton signal increases to 4.5 Hz.

Two factors could be contributing to the differential line broadening observed in 3,5-dimethyl-4-hydroxycinnamic acid at pH (pD) = 12.00. First, there could be exchange broadening, a contribution to the linewidth caused by the process of chemical exchange itself. Second, the differential broadening could be due to a greater transverse relaxation rate of the aromatic and β protons of the bound substrate relative to that of the α protons. This could occur if the orientation of the substrate protons in the complex is such that the aromatic and β protons are closer to particular protons of cyclohexaamylose causing them to experience enhanced nuclear dipole relaxation relative to the relaxation rate of the α protons. Although this is currently under investigation, we do not presently have sufficient information to assign the observed broadening to either of these mechanisms.

Cycloamylose Substrate Binding and Charge

A comparison of the binding of 3,5-dimethyl-4-hydroxybenzoic acid and 3,5-dimethyl-4-hydroxycinnamic acid in the cyclohexaamylose cavity at pH (pD) = 12.00, i.e., in the form of their dianions (Table 1), clearly demonstrates the importance of the position of the carboxylate anion in the cavity. With both substrates there is an increase in charge density on the carboxylate anion at pH (pD) = 12.00 relative to pH (pD) = 7.60. The depth of penetration of the benzoate carboxylate is clearly limited by the 3- and 5-methyl groups. The cinnamate carboxylate can penetrate the cycloamylose deep enough to hydrogen bond either the 6-OH groups or water molecules at the back of the cavity while the benzoate cannot. Furthermore, at pH 12.00 the 2-OH groups are at least partially ionized which would render any geometry B complex unstable. A geometry in which the carboxylate and a hydroxyl oxanion interact would clearly be a high-energy geometry. The pH 12.00 data then can be understood if geometry B is of importance in 3,5-dimethyl-4-hydroxybenzoate binding at pH 7.60.

CONCLUSION

It is apparent that 3,5-dimethyl-4-hydroxybenzoic acid binds in the cyclohexaamylose cavity at pH = 7.60, but not at pH = 12.00, while both the geometries and stabilities of the 3,5-dimethyl-4-hydroxycinnamic acid and 3,5-dimethyl-4-hydroxyhydrocinnamic acid cyclohexaamylose complexes are only marginally pH sensitive. These facts strongly suggest that the position of the carboxylate anion in the cyclohexa-

amylose cavity is of great importance in binding. This implies that carboxylate anion solvation is probably the single most important factor in regulating both the geometries and stabilities of cyclohexaamylose carboxylate anion complexes.

APPENDIX A

It is possible to measure the dissociation constant, and thus the free energy of formation, for cyclohexaamylose substrate complexes (CS) by observing changes in either the chemical shifts of substrate protons, or in the ultraviolet absorbance spectrum of the substrate (S) as a function of varying cycloamylose (C) concentration. Consider the following equilibrium expressions:

$$\mathrm{CS} \overset{K_D}{\rightleftharpoons} \mathrm{C} + \mathrm{S},$$

$$K_D = [\mathrm{S}][\mathrm{C}]/[\mathrm{CS}], \tag{1}$$

$$[\mathrm{C}]_0 = [\mathrm{C}] + [\mathrm{CS}], \tag{2}$$

$$[\mathrm{S}]_0 = [\mathrm{S}] + [\mathrm{CS}], \tag{3}$$

$$K_D = \frac{([\mathrm{S}]_0 - [\mathrm{CS}])([\mathrm{C}]_0 - [\mathrm{CS}])}{[\mathrm{CS}]}. \tag{4}$$

Which rearranges to:

$$\frac{[\mathrm{S}]_0\,[\mathrm{C}]_0}{[\mathrm{CS}]} + [\mathrm{CS}] = [\mathrm{C}]_0 + [\mathrm{S}]_0 + K_D. \tag{5}$$

In the case of uv absorption measurements, the observed absorbance is given by:

$$\mathrm{Abs} = [\mathrm{CS}]\,\varepsilon_{cs} + [\mathrm{C}]\,\varepsilon_c + [\mathrm{S}]\,\varepsilon_s. \tag{6}$$

Combining equations (2), (3), and (6) and rearranging gives:

$$\mathrm{Abs} = [\mathrm{CS}](\varepsilon_{cs} - \varepsilon_c - \varepsilon_s) + [\mathrm{C}]_0\,\varepsilon_c + [\mathrm{S}]_0\,\varepsilon_s. \tag{7}$$

The change in absorbance due to complexation is given by:

$$\Delta\mathrm{Abs} = \mathrm{Abs} - [\mathrm{C}]_0\,\varepsilon_c - [\mathrm{S}]_0\,\varepsilon_s. \tag{8}$$

Combining equations (7) and (8) and solving for [CS] gives:

$$[\mathrm{CS}] = \Delta\mathrm{Abs}/(\varepsilon_{cs} - \varepsilon_c - \varepsilon_s) = \Delta\mathrm{Abs}/\Delta\varepsilon, \tag{9}$$

where $\Delta\varepsilon \equiv (\varepsilon_{cs} - \varepsilon_c - \varepsilon_s)$. Combining equations (5) and (9) and dividing by $\Delta\varepsilon[\mathrm{S}]_0$ gives:

$$\frac{[\mathrm{C}]_0}{\Delta\mathrm{Abs}} + \frac{\Delta\mathrm{Abs}}{[\mathrm{S}]_0\,\Delta\varepsilon^2} = \frac{[\mathrm{C}]_0 + [\mathrm{S}]_0 + K_D}{[\mathrm{S}]_0\,\Delta\varepsilon}. \tag{10}$$

Thus, by plotting $[\mathrm{C}]_0/\Delta\mathrm{Abs} + \Delta\mathrm{Abs}/[\mathrm{S}]_0\Delta\varepsilon^2$ vs $[\mathrm{C}]_0 + [\mathrm{S}]_0$, a straight line with intercept equal to $K_D/[\mathrm{S}]_0\Delta\varepsilon$ and slope equal to $1/[\mathrm{S}]_0\Delta\varepsilon$ is obtained, which serves to

define both parameters K_D and $\Delta\varepsilon$. Initially, a plot of $[C]_0/\Delta Abs$ vs $[C]_0 + [S]_0$ [analogous to a Hildebrand–Benesi plot (*10*, *30*)] is performed to give an approximate value for $\Delta\varepsilon$. The successive approximations to the actual value of K_D and $\Delta\varepsilon$ are obtained by performing the plot indicated above until the value of $\Delta\varepsilon$ used in generating the $\Delta Abs/[S]_0\Delta\varepsilon^2$ form is sufficiently close to the value of $\Delta\varepsilon$ calculated from the slope of the line.

In the case of nmr measurements, the chemical shift is given by:

$$\delta_{obs} = \delta_s + \frac{[CS]}{[S]_0}(\delta_{cs} - \delta_s). \tag{11}$$

The change in chemical shift is given by:

$$\Delta\delta = \delta_{obs} - \delta_s. \tag{12}$$

Combining equations (11) and (12) gives:

$$\Delta\delta = \frac{[CS]}{[S]_0}(\delta_{cs} - \delta_s) = \frac{[CS]}{[S]_0}Q \tag{13}$$

where $Q \equiv (\delta_{cs} - \delta_s)$. Solving for [CS] gives:

$$[CS] = \frac{\Delta\delta[S]_0}{Q}. \tag{14}$$

Combining equations (14) and (5) and dividing by Q gives:

$$\frac{[C]_0}{\Delta\delta} + \frac{\Delta\delta[S]_0}{Q^2} = \frac{[C]_0 + [S]_0 + K_D}{Q}. \tag{15}$$

Thus, by plotting $[C]_0/\Delta\delta + \Delta\delta[S]_0/Q^2$ vs $[C]_0 + [S]_0$, a straight line with intercept equal to K_D/Q and slope equal to $1/Q$ is obtained, which serves to define both parameters K_D and Q. Treatment of nmr data this way is strictly analogous to that described above for uv data except that the product $\Delta\varepsilon[S]_0$ is replaced by Q.

ACKNOWLEDGMENTS

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Note added in proof. Laufer *et al.* have suggested that the benzoic acid is penetrating the cyclohexaamylose at the 6-hydroxy side, carboxyl group first (*31*). Their geometry is based on complexation-induced chemical shift changes in cycloamylose's C-6 and C-3 ^{13}C signals, the origins of which they suggested were singular in nature. These authors chose to completely neglect the direction in which the H-3 and H-5 protons shift on benzoic acid complexation and suggested the lack of shift in the C-6 methylene protons was understandable because of "complicated" rotamer distributions. We find that the direction and magnitude of the H-3 and H-5 shift, coupled with the fact that the C-6 methylenes do not shift (*16*) in the benzoic acid complex but have been shown to shift in other complexes (*32*) somewhat difficult to explain in terms of Laufer's proposed geometry. Furthermore, we feel that cycloamylose

substrate geometry assignments based solely on ^{13}C shifts are somewhat tenuous when the origins of the changes are unclear. For example, in an earlier ^{13}C of the *p*-nitrophenol and sodium *p*-nitrophenolate cyclohexaamylose we also observed the C-6 moving on complexation and were tempted to attribute this observation to *p*-nitrophenol penetration at the 6-OH side of the cavity (*33*). However, intermolecular nuclear Overhauser enhancements, ^{1}H chemical shift studies and an X ray of the complex made it clear that such ^{13}C data can be easily misinterpreted. Consequently, we are compelled to interpret our findings in terms of our original structure until more definitive, less speculative evidence is available.

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